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CHARACTERIZATION AND DIFFERENTIATION OF THE "INTERMEDIATES" OF THE COLON-AEROGENES GROUP OF BACTERIA

BY

Nancy Booth Mitchell

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Appreciation is expressed to Dr. Max Levine for his constructive criticism.

I. INTRODUCTION

The colon group of bacteria may be characterized as "non-sporing, Gram negative bacilli which ferment lactose with the production of acid and gas and which are capable of growing aerobically" (Levine 1921). Their normal habitat is the intestinal tract of warm blooded animals and the soil. Emmerich (1884) first isolated a member of this group from the feces of a case of cholera infantum.

There are at the present time two universally accepted genera, Escherichia and Aerobacter, and a third proposed genus, Citrobacter.

Aerobacter aerogenes (Escherich) Beijerinck differs from Escherichia coli (Escherich) Castellani and Chalmers in that it: produces appreciable quantities of acetyl-methyl-carbinol from glucose; utilizes the citrate radical as a sole source of carbon; utilizes uric acid as a sole source of nitrogen; and decomposes approximately 14% of glucose into acids, as compared to approximately 56% decomposed by E. coli.

The status of the proposed genus Citrobacter is not settled. The validity of the genus has not been accepted by the majority of workers, and the definition of the term "intermediate group" varies with the different authors. For

these reasons a more complete historical review of this group was considered desirable.

In 1923 Koser showed that strains of the genus Aerobacter could utilize citric acid as a sole source of carbon, whereas strains of the genus Escherichia could not. The following year he investigated 72 coliform strains isolated from unpolluted soil and found that 25 were MR (+), VP (-)*. Of these 25 strains, 23 were also citrate (+); most of these strains were also uric acid (-). Due to their source he referred to them as "soil forms" of Escherichia. The possible sanitary significance of this finding was immediately realized by sanitary bacteriologists. The relationship of habitat to morphology and biochemical characteristics was, and still is, the chief hope for a criterion of water pollution.

Workers supporting the idea that citrate (+) coli-aerogenes forms are not indicative of dangerous pollution are: Koser (1924-a, 1924-b); Pawan (1931); Raghavachari (1926); Hicks (1927); Lewis and Pittman (1928); Winkewitsch (1930);

* Throughout:
MR (+) signifies production of acid reaction to methyl red.
VP (+) signifies production of acetyl-methyl-carbinol.
Citrate (+) signifies availability of carbon of citrate radical.
Uric acid (+) signifies availability of nitrogen of uric acid.
The reverse symbol (-) signifies lack of same characteristics.

Wolwerde (1930); Brown and Skinner (1930); Ruchhoft et al (1931); Burke-Gaffney (1932); and Bardsley (1934). Reports to the contrary have been made by: Taylor et al (1927); Gray (1932); and Tittsler and Sandholzer (1935-a).

Bardsley (1934) investigated the distribution and sanitary significance of coliform bacilli in England. Her summary of previous work was justified: "Most workers in tropical countries where pollution is very heavy find it practical to distinguish between the different types of coliform bacilli, and only those organisms which conform to the true E. coli group (MR (+), citrate (-)) are regarded as indicators of excretal contamination....In temperate climates the position is not so well defined."

Bardsley's investigation of 5131 coliform strains (4333 from water, 153 from soil, 331 from feces, and 365 from ice cream) showed that "intermediate" organisms were dominant in soil, were frequently isolated from ice cream, but were rare in water and in feces. She considered "intermediate" organisms to be MR (+), VP (-), indol (-), uric acid (-), citrate (+). Her conclusion: "The occurrence of aerogenes and the intermediate type in food and water supplies (in England) may be due to fecal pollution, although the presence of these bacteria in the absence of B. coli would seem to suggest that the pollution has not been recent."

In 1938 Braak investigated the dissimilation of glycerol by the colon group. He found that two strains which were capable of an anaerobic fermentation of glycerol in a purely mineral medium produced varying amounts of trimethylene glycol from glycerol. These two strains were MR (+), VP (-), citrate (+), indol (+); one strain was uric acid (+) and the other one uric acid (-). Typical cultures of E. coli, A. aerogenes, or A. cloacae did not produce trimethylene glycol. The determination of the trimethylene glycol was by a lengthy chemical procedure which involved isolation, purification, and identification of the compound.

Gillen (1940) investigated MR (+), VP (-), citrate (+) organisms which were capable of growing anaerobically in a mineral glycerol medium. Nine out of eleven such strains produced trimethylene glycol from glycerol. Davis (1941) found that 31 "representative cultures" of MR (+), VP (-), citrate (+) strains produced trimethylene glycol from glycerol. Tarnutzer (1952) showed that 34 out of 41 MR (+), VP (-), citrate (+) strains produced the glycol from glycerol.

In all, 64 MR (+), VP (-), citrate (+) strains have been shown to ferment glycerol with the production of trimethylene glycol. Their characteristics, as far as given, were:

Investigator	Strains	Uric		Indol		H_2S	
		+	-	+	-	+	-
Gillen	9	1	8	4	5	7	2
Davis	21	12	9	1	20		
Tarnutzer	34	0	34	0	34	34	0

In 1932 Werkman and Gillen proposed the generic name Citrobacter. They stated: "The diagnosis will not be limited, for the present, to organisms producing trimethylene glycol although production of glycol occurs with all the cultures so far examined.

Diagnosis: Gram-negative, non-sporulating short rods with rounded ends. Methyl-red positive or intermediate. Acetoin not produced from glycerol and rarely from glucose and then only in traces. Citrate radical utilized. Nitrates reduced to nitrites. Many carbohydrates, alcohols and glucosides attacked with the production of acid and gas. Ammonium salts utilized as a source of nitrogen. Type species: The type species is Citrobacter Freundii (Braak) comb. nov."

Seven species were differentiated on the basis of gelatin liquefaction and fermentation of certain sugars. Cit. Freundii produced slight indol, produced hydrogen sulphide, and did not assimilate uric acid. Two strains gave weak VP tests on the fifth day.

Koser (1936) found that many MR (+), VP (-),citrate (+) strains fermented cellobiose with the production of acid and gas. He stated that typical E. coli strains (VP (-), citrate (-)) should not be considered indices of pollution if they fermented cellobiose, but such strains should be classified with the "intermediate" VP (-), citrate (+) group. Skinner and Brudnoy (1938) found little or no correlation between cellobiose fermentation and citrate utilization.

Levine, et al (1932) showed that 100% of 43 "intermediate" strains produced hydrogen sulphide in a special solid medium, whereas less than 1.0% of 358 typical Escherichia and Aerobacter strains did so. Later these workers called attention to the importance of the concentration of agar in the determination of hydrogen sulphide. This, in the main, has been substantiated by further work: Levine, Epstein, and Vaughn (1934), Tittsler and Sandholzer (1935-a) and Vaugh and Levine (1936).

Koser and Saunders (1932) found that fermentation of alpha-methylglucoside differentiated the Escherichia and Aerobacter genera, but was of slight or no value for the MR (+), VP (-) citrate (+) strains. Tittsler and Sandholzer (1935-b) found that of 69 "intermediate" cultures tested, 61 fermented cellobiose, 43 utilized citrate, and 20 fermented alpha-methylglucoside.

Tittsler and Sandholzer (1935-a) presented a detailed

study of the "intermediate" group. They characterized it: "This group resembles the genus Escherichia in that it is MR (+), VP (-), and produces either a colon or colon-like type of colony on eosin methylene blue agar. In other respects it resembles the genus Aerobacter in that many of the cultures utilize citric acid as a sole source of carbon and ferment cellobiose, while some are citrate positive and cellobiose negative..... The production of hydrogen sulphide will serve, however, as a differential characteristic for some of the cultures."

Four characteristics were used to separate the 29 strains into six major groups as shown in the following table:

Number of strains	9	4	8	6	1	1
Cellobiose	(+)	(+)	(+)	(+)	-	-
Citrate	+	+	+	-	+	-
H_2S	+	-	+	-	-	+
Alpha-methyl-glucoside	(+)	(+)	-	-	-	-

For Sugars

(+) indicates production of acid and gas
\+ indicates production of acid only
\- indicates neither acid nor gas produced

The strains were allocated to the genus Escherichia as there was found no single characteristic (with the possible exception of production of trimethylene glycol or hydrogen sulphide) which would separate them from the genera Escherichia and Aerobacter.

Whether the "intermediate" group constitutes a physiologically homogeneous unit distinct from Aerobacter and Escherichia is undetermined. Recent work has shown these organisms to occur more frequently in soil than in feces, although they are certainly present, at least in small numbers, in the latter.

The characterization of the genus Citrobacter as "methyl red positive or intermediate. Acetoin.... produced rarely from glucose and then only in traces," does not distinctly separate it from the genera Escherichia and Aerobacter, but instead constitues a division for organisms giving borderline reactions. If a large number of citrate (+), VP (?) and questionable MR strains, as well as MR (+) and VP (-) strains, could be shown to produce the trimethylene glycol, then the genus would have a characteristic clearly separating it from closely allied strains. To be of value to systematic bacteriology such a test would have to incorporate a simple and rapid method. Further work would have to be done to establish the inability of Escherichia and Aerobacter strains to produce the glycol.

If, on the other hand, there are amassed data of sufficient statistical bearing to indicate that the MR (+), VP (-), citrate (+) strains, as were first isolated by Koser (1924), constitute a homogeneous group from the standpoint of correlated biochemical characters, then the argument for the creation of a genus is strengthened. "Individual characters are not considered paramount and independently, but only in relation to each other." (Levine 1921). But should such a correlation study, although unified within itself, show a decidedly greater similarity to the Escherichia or Aerobacter, then the inclusion of the group within that genus would be indicated.

Because of differences in technique it is difficult to assess the value of any study of correlation of characters. It is even more difficult when the definitions of "intermediate" vary.

Koser (1924) first described organisms of the colon group which were MR (+), VP (-), and citrate (+). A majority of workers allocate coli-forms giving these reactions to the "intermediate" group. One may accept, therefore, these three characteristics as definitive of the broad use of the term "intermediate."

Koser (1926), and Tittsler and Sandholzer (1935-a) have employed a still broader definition in that they considered colon organisms which were citrate (-), cellobiose (+) should

be grouped with the "intermediates." Bardsley (1934), in a valuable statistical study, found 597 MR (+), VP (-), citrate (+), uric acid (-) strains. Of these, 581, or 97.3%, were also indol (-). She, therefore, included the indol (-) reaction as a necessary criterion for the "intermediate" group.

Koser (1924-c) found that organisms did not readily acquire or lose the ability to utilize citric acid as a sole source of carbon. Minkewitsch (1930) failed to produce citrate utilizing strains from cultures of typical B. Coli even though he subjected a large number of strains to many different environments.

It is apparent that the physiological characterization of the colon group has been stressed from the standpoint of carbohydrate chemistry. The ability of these organisms to utilize ammonia as a sole source of nitrogen, whereas they are unable to utilize the carbon dioxide of the atmosphere; the necessity of chemically clean glassware; and the lack of any easily applied test for chemical changes, such as is provided in the production of acid and gas from carbohydrate dissimilation, have made the study of the nitrogen chemistry a far less developed field.

Work with several purine compounds provides an exception. Plenge (1903) and Schittenholm and Schröter (1903,1904) presented evidence that B. coli attacked nucleic acid. Koser (1918) showed that uric acid was utilized by

Aerobacter strains as a sole nitrogen source, but not by Escherichia. At the same time he noted that hypoxanthine hydrochloride gave the same differentiation, although A. aerogenes strains grew less luxuriantly. Chen and Rettger (1920) confirmed Koser's work on uric acid but found that of 20 coli-like strains from soil, 10 were uric acid (+), and 10 were uric acid (-). They found that xanthine gave the same results as uric acid with typical E. coli strains, but that of the 20 soil forms, all were xanthine (-).

Glucose metabolism in muscle and the process of alcoholic fermentation by yeast correlates work in bacteriology and biochemistry. Uric acid, used as a sole nitrogen source, differentiates the genera Escherichia and Aerobacter. Uric acid is an end product of nucleic acid digestion in man and anthropoid apes; allantoin is the end product in most other animals. Allantoin is oxidized, in vitro, to hydantoin and urea, and further to glycine, carbon dioxide, and ammonia. It appeared, therefore, that a study of nucleic acid and its degradation products, when fermented by the colon group from the standpoint of the nitrogen compounds attacked, might prove significant. At the same time investigations were carried out to determine the possibilities of pyruvic acid fixation in a glycerol-peptone-bisulphite medium as a means of differentiating the "intermediate" group from the genera Escherichia and Aerobacter as suggested by Reynolds (1935).

II. EXPERIMENTAL

A. Source of Cultures

The source of cultures is given in Table I. Appreciation is expressed to the contributors.

Table I.

Source of Cultures

Group	Total Strains	Source	No. Strains	Contributed by	Isolated or Received
Escherichia	106	Frozen eggs	95	D.Q.Anderson	1929
		Surface water	2	S.S.Epstein	1933
		Animal feces	2	S.S.Epstein	1933
		Human feces	2	S.S.Epstein	1933
		Fowl feces	1	S.S.Epstein	1933
		American Type C.C.	4		
"Intermediates"	138	Frozen eggs	48	D.Q.Anderson	1929
			1	C.H.Werkman	1932
		Human feces	9	S.S.Epstein	1933
		Fowl dejecta	3	S.S.Epstein	1933
		Shucked oysters	1	S.S.Epstein	1933
		Rotten potato	1	S.S.Epstein	1933
			16	C.E.Skinner	1933
		Swimming pool water	9	D.A.Bardsley	1934
			3	S.A.Koser	1935
			9	H.Reynolds	1935
			11	R.P.Tittsler	1935
			3	M.M.Barritt	1936
			16	R.L.Carpenter	1936
		Ice cream	1	M.Grimes	1936
			2	A.J.Kluyver	1936
			2	J.Smit	1936
			3	G.S.Wilson	1936
Aerobacter	111	Frozen eggs	86	D.Q.Anderson	1929
		Soil	2	S.S.Epstein	1933
		Chicken dejecta	2	S.S.Epstein	1933
		Rotten potato	2	S.S.Epstein	1933
		Surface water	5	S.S.Epstein	1933
		Human feces	14		1934
VP (?)	6	Frozen eggs	1	D.Q.Anderson	1929
		Human feces	2	S.S.Epstein	1933
			1	H.Reynolds	1935
			1	M.M.Barritt	1936
			1	A.J.Kluyver	1936

B. Characteristics of Organisms Employed

The Escherichia and Aerobacter strains were checked for lactose fermentation, appearance on eosin-methylene-blue agar, gram stain, acetyl-methyl-carbinol production, and utilization of the citrate radical as a sole source of carbon. Only strains which were typical in all these respects were employed.

Additional characteristics of the Escherichia and Aerobacter strains were not checked as the present investigation was primarily concerned with the differentiation and characterization of the "intermediate group." Data compiled for these same strains by other workers in this laboratory were available, however. The 106 Escherichia cultures included representatives of sucrose (+) and (-), salicin (+) and (-), and dulcitol (+) and (-) strains. The 111 Aerobacter strains included: 39 A. cloacae; 31 A. aerogenes, indol (+); and 41 A. aerogenes, indol (-). The collection was considered representative for the genera in question.

The "intermediate" strains were purified by inoculation into lactose broth, followed by streaking on to eosin-methylene blue agar. Colonies were picked and seeded into lactose broth. After fermentation the culture was again streaked out on the differential medium. Those strains showing homogeneous colonies on eosin-methylene-blue agar after three such series

were considered pure. Some strains still showed heterogeneous colonies after six platings. It was considered impractical to continue plating these, and they were checked for other characteristics.

The six VP (?) strains were received at this laboratory as "intermediate" organisms and consequently were purified as outlined above. Subsequently they were found to give a questionable test for acetyl-methyl-carbinol and, therefore, were placed tentatively in a separate group.

The following characteristics were ascertained for the purified "intermediate" and the six VP (?) strains: acetyl-methyl-carbinol production, citrate utilization, methyl red reaction, cellobiose fermentation, hydrogen sulphide production, indol production, nitrate reduction, liquefaction of gelatin, and utilization of uric acid as a sole source of nitrogen.

All tests were carried out in the following manner.

VP: acetyl-methyl-carbinol production was determined by heavy inoculation from a young agar culture into 1.5 ml. of Difco "LR-VP Medium." After 24 hours' incubation at 30° C. Barritt's alpha-naphthol test (1936) was applied (Coblentz, 1937). All of the "intermediate" strains employed were VP (-).

Citrate test: the utilization of citric acid was tested in Difco "Koser citrate medium." The inoculum consisted of a one mm. loop of young nutrient broth culture. Results

were recorded after five day's incubation at 37° C. All of the "intermediates" employed and the 6 VP (?) strains were citrate (+).

Methyl red: Difco "MR-VP Medium" was used. Inoculations were made into five ml. of medium in 18 mm. test tubes. After five days' incubation at 30° C. five drops of methyl red were added. Practically all intermediate strains were MR. (+). Seven strains were MR (-) and four were MR (±). These were included as "intermediates" on the basis of the VP and citrate tests; also, previous investigators in this laboratory had recorded them as MR (+) organisms.

Cellobiose: the medium consisted of standard nutrient broth plus 0.3% cellobiose and 0.004% brom-thymol-blue. Incubation was at 30° C. for five days. Of the 138 "intermediate" strains, seven produced neither acid nor gas, 87 produced acid but no gas, and 34 produced both acid and gas.

Hydrogen sulphide: production of hydrogen sulphide was tested according to Vaughn and Levine (1936). The medium was composed: 3.0% proteosepeptone (Difco), 1.5% agar (Difco), 0.1% K_2HPO_4, and 0.05% ferric citrate. Incubation was at 37° C. for four days. Eighty-three per cent of the "intermediate" strains produced hydrogen sulphide. The six VP (?) strains were H_2S (-).

Indol: production of indol was tested in 1% Difco tryptophane (hydrolized casein). After five days' incubation at 37° C. Kovac's reagent (1928) was added. One hundred thirty, or 94%, of the "intermediate" and five of the VP (?) strains were indol (-).

Nitrate reduction: the test medium was composed of 0.8% peptone (Difco), 1.0% Na Cl, and 1.0% K NO_3. After two days' incubation at 37° C. the sulfanilic acid: naphthylamine-acetate test was applied. All of the "intermediate" and VP (?) reduced nitrates to nitrites.

Gelatin liquefaction: the test medium was composed of 0.3% beef extract, 0.5% peptone (Difco), and 15.0% gelatin (Difco). Incubation was at 20° C. for five weeks. None of the "intermediates" or VP (?) strains liquefied gelatin.

Uric acid: Koser's medium (1918) was employed. The inoculum consisted of a one mm. loop of young nutrient broth culture. Vigor of growth was recorded after five days at 30° C. All of the "intermediates" were uric acid (-). The six VP (?) strains were uric acid (+).

The individual characteristics of the "intermediate" and VP (?) strains appear in Table A. of the appendix.

C. Determination of Utilization of Nitrogen Compounds

Yeast nucleic acid and a number of its degradation products were investigated as nitrogen and carbon sources for the colon group. The investigation dealt primarily with the study of the nitrogen availability of these compounds in an effort to secure a broad approach, other than carbohydrate chemistry, for ascertaining the systematic relationships of the colon group. The investigations of these compounds as carbon, or combined carbon and nitrogen sources, was of a survey nature only. It was undertaken in order to secure some indication as to the possible value of a further, and different, investigation of these compounds.

The compounds studied were: yeast nucleic acid, adenine sulphate, guanine hydrochloride, xanthine, uric acid, uracil, allantoin, hydantoin, and urea. All were Eastman products except urea.

1. Methods

To test the availability of a compound as a nitrogen source, Medium I was employed. The determination of utilization of nitrogen was judged by two criteria: (1) acidification of the medium, as it was assumed that the glucose would be attacked appreciably, thus creating an acid reaction, only if there were an available and adequate source of nitrogen;

and (2) vigorous growth, as is employed in the Koser (1918) medium.

Medium I

To test availability of compound as nitrogen source.

Na Cl	0.5%
$MgSO_4$	0.02%
Dextrose	0.2%
Test compound	0.05%
Brom-thymol-blue	0.004%

1/m phosphate buffer (p.H 7.1) 2.0% (by volume)

To determine the availability of the carbon of the test compound, an ammonium salt was supplied as a source of nitrogen, and glucose from Medium I omitted. Luxuriant growth was recorded as evidence of an available carbon supply. In some cases the decomposition of the test compound itself led to an acidification of the medium.

Medium II

To test availability of compound as carbon source.

Na Cl	0.5%
$MgSO_4$	0.02%
$(NH_4)_2HPO_4$	0.2%
Test Compound	0.05%
Brom-thymol-blue	0.004%

1/m phosphate buffer (pH 7.1) 2.0% (by volume.)

To investigate the ability of the test compounds to serve as sole sources of both carbon and nitrogen, Medium III was employed. Vigorous growth was recorded as evidence of available carbon and nitrogen.

Medium III
To test availability of compound as carbon and nitrogen source

Na Cl	0.5%
$Mg\ SO_4$	0.02%
Test compound	0.5%
Brom-thymol-blue	0.004%
1/m phosphate buffer (pH 7.1)	2.0% (by volume)

All glassware coming in contact with the media was previously washed in acid-dichromate solution, rinsed in sodium bicarbonate solution, and then rinsed three times in distilled water.

Conductivity water was employed as the solvent in all media. The constituents were put into solution at a temperature below boiling. Phosphate buffer ($K_2\ HPO_4$-$Na\ H_2\ PO_4$) was used to the extent of 2.0% by volume unless this was found insufficient to adjust the pH to approximately 7.1; in such a case more of the necessary solution was added. A five day preliminary run on allantoin, in which the pH was determined electrometrically, suggested the use of brom-thymol-blue as an indicator.

Since the effect of autoclaving was unknown, the media were sterilized by filtration through Chamberlain candles of L3 or L5 porosity; the candles were incinerated after each run. The media were tubed aseptically and incubated 3-6 days at 30° C. to test for sterility.

Since further work would be facilitated if the media could be sterilized by autoclaving, the effect of moderate autoclaving (15 pounds for 12 minutes, followed by immediate cooling) was tested in an exploratory manner.

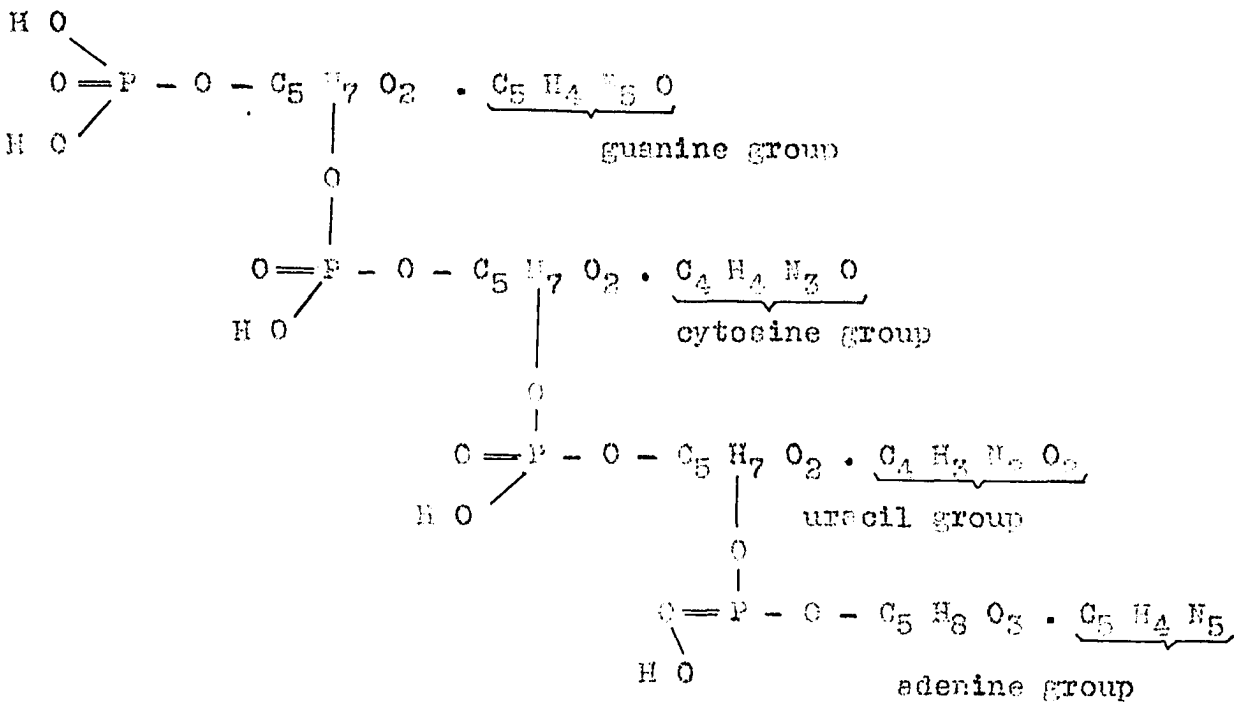
Incubation was at 28.5-30° C. (temperature of the medium). Inoculations consisted of a one mm. loop of a 24 hour nutrient broth culture.

2. Results

As will be evident from the data for uric acid, it was found that the indirect test for nitrogen utilization, i.e. acidification of the medium due to glucose dissimilation, correlated quite well with the accepted criterion of vigorous growth. The following results are presented stressing the indirect test, but in all cases the data for vigor of growth are given for comparison.

a. Experiments with purine compounds. The following purine or purine containing compounds were investigated: yeast nucleic acid, adenine sulphate, guanine hydrochloride, xanthine and uric acid.

(1) Yeast nucleic acid. Levene (1920) gives the following molecular configuration for yeast nucleic acid:



yeast nucleic acid

The carbohydrate component is d-ribose.

The nucleic acid went into solution easily upon heating. Excessive heating caused the medium to become more acidic.

When nucleic acid in medium (No. 1) was inoculated with 103 Escherichia, 138 "intermediate," and 110 Aerobacter strains, it was found that the nitrogen was fairly readily available to the Aerobacter strains, but much less so to the other groups. Table No. II gives the results of the indirect test for the utilization of the nitrogen of this compound by the colon group.

Table II

Acid Production from Glucose with Nucleic Acid Supplied as Nitrogen Source (28-30° C)

Group	Total Number of Strains	Per Cent of Strains Acidifying Medium				
		1 day	2 day	3 day	4 day	5 day
Escherichia	103	0	0	0	0	32
"Intermediate"	138	0	0	0	0	47
Aerobacter	110	29	51	86	94	99
VP (?)	6	100	100	100	100	100

The acidification of the medium by the Escherichia and "intermediate" strains on the fifth day was very slight. None of the strains reversed the reaction, after having once acidified the medium.

A study of Table II shows that: (1) such a medium would have value as a differential test when a short incubation period (four days or less) was employed; (2) the VP (?) strains behaved as the genus Aerobacter, except that they acidified the medium even more promptly.

A closer study of the data revealed that of the 39 A. cloacae strains, 56% had slightly acidified the medium on the first day, as compared to 13% of the 71 A. aerogenes strains. On the third day the percentage of either species still failing to attack the glucose was approximately the

same, (15%), but of the strains that did attack the sugar 41% of the A. cloacae strains as compared to 27% of the A. aerogenes strains had produced enough acid to lower the pH to at least 6, i.e. yellow color range of brom-thymol-blue. The A. cloacae species, therefore, may be considered as utilizing the nitrogen of yeast nucleic acid more readily than the A. aerogenes species. There was no difference in the indol (+) and (-) groups of A. aerogenes.

None of the four groups of Table II grew vigorously, although the turbidity produced by the Aerobacter and VP (?) strains was greater than that produced by the other two groups.

An exploratory run on nucleic acid as the sole source of carbon (Medium No. II) was made with ten Escherichia, eight "intermediate," five A. cloacae, and five A. aerogenes strains. Only the five A. aerogenes strains, however, showed a fairly heavy growth and a slight acidification of the medium after five days.

A similar exploratory run was made on this compound as the source of both carbon and nitrogen (Medium No. III) with the same strains. Again only the five A. aerogenes strains grew at all well; there was no appreciable change in reaction.

The nucleic acid medium (No. I) was subjected to moderate autoclaving and then inoculated with the 28 test organisms (ten Escherichia, eight "intermediate," five A. cloacae

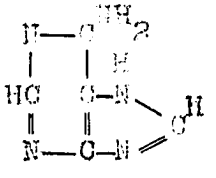
and five A. aerogenes strains). Only two strains, one Escherichia and one Aerobacter, gave results different from that obtained with the filtered medium. Apparently moderate autoclaving did not cause any material breakdown of this compound.

In the synthetic medium (No. I), yeast nucleic acid was a readily available nitrogen source for the Aerobacter and VP (?) strains, but not for Escherichia or "intermediate" strains. With a four day incubation period at 25-30° C. the medium appeared to have differential value for separating the A. aerogenes from the other strains of the colon group.

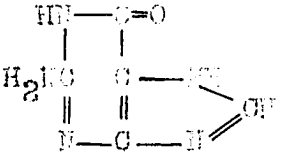
In exploratory runs, employing a few organisms, made with nucleic acid as a carbon, or carbon and nitrogen, source only A. aerogenes strains grew moderately well.

There was evidence that moderate autoclaving did not appreciably affect this compound.

(2) Adenine sulphate and guanine hydrochloride



adenine



guanine

The positions of the sulphate and hydrochloride radicals of these purines were not stated for the Eastman products.

The adenine sulphate went into solution easily upon heating. As this compound acidified the medium, additional basic phosphate solution was added.

All compounds investigated were first subjected to a preliminary investigation to determine if such a medium would permit the growth of any of the colon group.

The preliminary experiment with adenine sulphate showed that when ten Escherichia, eight "intermediate," and ten Aerobacter strains were inoculated into adenine sulphate medium (No. I), that all strains grew abundantly and strongly acidified the medium by the second day. Complete reversion to the original reaction had occurred with eight Escherichia, three "intermediates," and three Aerobacter strains by the eleventh day. Since there was no differential action shown, an investigation of a greater number of strains was not undertaken.

An exploratory run was made on adenine sulphate as a sole source of carbon (Medium No. II). Of the ten Escherichia, eight "intermediates," five A. cloacae, and five A. aerogenes strains inoculated, only the A. aerogenes grew vigorously. The A. aerogenes strains had not changed the reaction of the medium after five days' incubation.

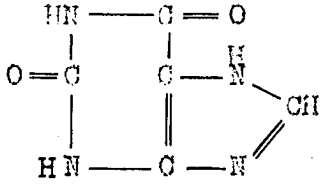
An exploratory run showed that as a sole source of both carbon and nitrogen (Medium No. III) the adenine sulphate failed to support the growth of any of the 28 test organisms.

Since this compound was not subjected to any detailed study, the effect of autoclaving was not determined.

Observation with a limited number of strains indicated that adenine sulphate was an available source of nitrogen for all of the strains tested (Escherichia, "intermediate," A. cloacae and A. aerogenes). Exploratory runs seemed to show that this compound behaved as nucleic acid as a source of carbon in that it supported the growth of A. aerogenes only; but that it differed from nucleic acid as a source of both carbon and nitrogen in that it failed to support the growth of any of the colon group.

It was found impossible to put the guanine hydrochloride into solution.

(7) Xanthine



Xanthine

The xanthine went into solution only upon prolonged heating. It was filtered while warm. After three days' incubation, to test for sterility, it was observed that some of the xanthine had come out of solution and floated on the surface of the liquid as a small, opaque, yellowish substance.

A preliminary investigation of xanthine (Medium No. I) on the third day of incubation showed that seven of the ten Escherichia, six of the eight "intermediates," and all of the ten Aerobacter strains had acidified the medium. As judged by the color change of the indicator the amount of acid produced by the Aerobacter strains was greater than that produced by the Escherichia or "intermediate." This was considered inconclusive, and a larger number of strains was used. Due to the expensiveness of the xanthine the total number of cultures was not employed.

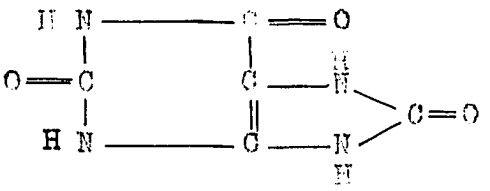
Xanthine medium (No. I) was inoculated with 50 Escherichia, 50 "intermediate," 50 Aerobacter and the 6 VP (?) strains. At least 96% of each group acidified the medium after 24 hours' incubation. All strains, except for one Escherichia, acidified the medium by the fourth day. There was some indication of beginning reversion on the fifth day.

All strains which changed the reaction grew vigorously.

The availability of xanthine as a sole source of carbon, or of both carbon and nitrogen, and the effect of autoclaving were not determined, due to the expensiveness of the compound.

Xanthine was similar to adenine sulphate, therefore, in that it was an available nitrogen source for all of the colon strains tested.

(4) Uric acid



uric acid

The uric acid went into solution fairly readily. The substitution of glucose for glycerol seemingly had no effect on the solubility, contrary to what Koser suggested (1924-a).

When 103 Escherichia, 138 "Intermediate," 110 Aerobacter, and the 6 VP (?) strains were inoculated into uric acid medium (No. I), it was found that the Aerobacter and VP (?) strains rapidly acidified the medium, whereas only 3% of either the Escherichia or "intermediate" strains had attacked the sugar appreciably by the fifth day of incubation. The tabulated results of this experiment appear in Table III.

In 1918 Koser showed that Aerobacter strains utilized the nitrogen of uric acid and grew vigorously, whereas Escherichia strains did not. Table III shows that acid production from glucose, when uric acid is the sole source of nitrogen, provides another method of testing this characteristic.

Table III

Acid Production from Glucose with Uric Acid Supplied as Nitrogen Source (28–30° C.)

Group	Total Number of Strains	Per Cent of Strains Acidifying Medium				
		1 day	2 day	3 day	4 day	5 day
Escherichia	103	0	0	0	0	3
"intermediate"	138	0	0	0	0	3
Aerobacter	110	76	93	96	100	100
VP (?)	6	100	100	100	100	100

The A. aerogenes utilized the nitrogen of this compound more readily than did the A. cloacae strains as judged by rapidity of acid production. Of the 72 A. aerogenes strains, 14 of the 31 indol (+) organisms, and one of the 41 indol (-) organisms had reversed the reaction to the original alkalinity by the fifth day.

None of the Escherichia or "intermediate" strains grew vigorously, whereas all of the VP (?) and 75% of the Aerobacter strains did so. Table IV shows the vigor of growth for A. cloacae and A. aerogenes strains in uric acid medium (No. I).

Table IV

Vigor of Growth in Uric Acid Medium No. I.

Species	Number of Strains	Per Cent Strains Showing Vigorous Growth		
		1 day;	2 day;	5 day
A. cloacae	39	31	31	31
A. aerogenes	72	95	100	100

That 27 of the A. cloacae strains failed to grow vigorously, although the medium was acidified, was noteworthy.

Uric acid (Medium No. II) was tested in an exploratory manner as a carbon source for ten Escherichia, eight "intermediate," five A. cloacae and five A. aerogenes strains. Only the A. aerogenes strains grew vigorously, and of these five strains, one slightly acidified the medium. When uric acid (Medium No. III) was tested as a source of both carbon and nitrogen for the same 28 strains, again only the A. aerogenes organisms grew vigorously, and the same strain acidified the medium.

Koser (1918) showed that autoclaving did not affect uric acid.

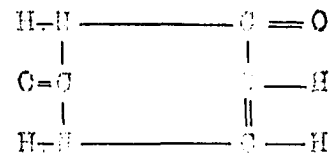
Uric acid was a readily available nitrogen source for Aerobacter and VP (?) strains but not for Escherichia and "intermediate" strains. In this it resembled nucleic acid.

The two compounds differed in that nucleic acid was attacked slowly by the Escherichia and "intermediate" strains (after five days), whereas uric acid was not.

Exploratory runs indicated that uric acid was an available carbon source, or both carbon and nitrogen, for A. aerogenes, only, of the colon strains tested. In this respect it resembled nucleic acid.

Koser (1918) showed that uric acid was not affected by autoclaving.

b. Experiments with a pyrimidine compound, uracil.



uracil

The uracil went into solution easily upon heating.

When a large number of Escherichia, "intermediate," Aerobacter, and the six VP (?) strains were inoculated into uracil medium (No. I), the results showed that the nitrogen of this compound was quite readily available to the Aerobacter and VP (?) strains, less so to the Escherichia, and practically unavailable to the "intermediate" strains. The tabulated data for this experiment appear in Table V.

Table V

Acid Production from Glucose with Uracil Supplied as Nitrogen Source (28-30° C.)

Group	Number of Strains	Per Cent of Strains Acidifying Medium				
		1 day	2 day	3 day	4 day	5 day
Escherichia	106	3	61	79	80	80
"Intermediate"	138	0	2	2	2	2
Aerobacter	111	76	99	100	100	100
VP (?)	6	33	100	100	100	100

None of the Escherichia strains had reversed the acid reaction by the fifth day. A few Aerobacter strains showed beginning reversion on the third day; complete reversion to the original alkalinity had not been obtained by any strain by the fifth day. The behavior of the A. cloacae, A. aerogenes indol (+), and A. aerogenes indol (-) strains was quite similar.

None of the Escherichia or "intermediate" strains showed vigorous growth in this uracil medium in five days, whereas 100% of the Aerobacter and VP (?) strains grew vigorously in two days.

In an exploratory run the uracil medium (No. II) was tested as to its availability as a carbon source for ten Escherichia, eight "intermediate," five A. aerogenes, and five A. cloacae strains. Only the five A. aerogenes strains grew vigorously, one of which slightly acidified the medium.

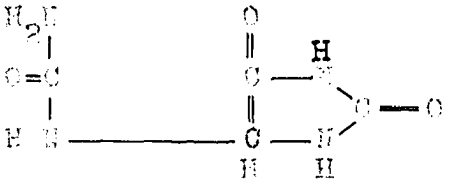
In an exploratory run to test uracil medium (No. III) as an available source of both carbon and nitrogen it was found that of 16 colon strains: five Escherichia, five "intermediate," and two A. cloacae strains neither grew vigorously nor changed the reaction; four A. aerogenes strains grew well, but not as luxuriantly as when glucose was supplied, and did not change the reaction.

When uracil medium (No. I) was subjected to moderate autoclaving, a test run showed that of 28 colon strains: the ten Escherichia, the ten Aerobacter, and two of the eight "intermediate" strains acidified the medium. Only the Aerobacter strains grew abundantly. It is probable that moderate autoclaving does not affect uracil.

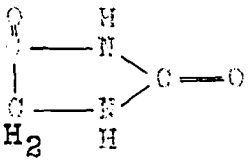
In the synthetic medium (No. I) uracil was an available nitrogen source for all of the Aerobacter and VP (?) strains, for 84 of 106 Escherichia strains, but for only two of 138 "intermediate" strains. Uracil, therefore, differed from the other compounds tested in that it differentiated to a high degree the "intermediate" group from the genera Escherichia and Aerobacter. Further investigation of this and other pyrimidine compounds as nitrogen sources is suggested as offering a possible means of differentiating the "intermediate" group from other colon strains.

Exploratory runs indicated that uracil behaved as nucleic acid and uric acid in that it served as a source of carbon, or carbon and nitrogen, for only A. aerogenes strains of the colon group. An exploratory run indicated that moderate autoclaving did not materially affect uracil.

c. Experiments with allantoin and hydantoin



allantoin



hydantoin

Both allantoin and hydantoin went into solution fairly easily upon heating.

A large number of colon strains were inoculated into hydantoin and allantoin, Medium No. I. Both compounds served as a nitrogen source for the Aerobacter and VP (?) strains, but failed as a nitrogen source for the Escherichia and "intermediate" strains. The results of these experiments appear in Table VI.

Table VI

Acid Production from Glucose with Allantoin or Hydantoin Supplied as Nitrogen Source

Group	Number of Strains	Per Cent of Strains Acidifying Medium				
		1 day	2 day	3 day	4 day	5 day
Allantoin as nitrogen source						
Escherichia	106	0	0	0	0	0
"Intermediate"	138	0	0	0	0	0
Aerobacter	110	77	95	97	100	100
VP (?)	6	100	100	100	100	100
Hydantoin as nitrogen source						
Escherichia	104	0	0	0	0	0
"Intermediate"	138	0	0	0	0	0
Aerobacter	111	69	80	86	87	95
VP (?)	6	100	100	100	100	100

When the indirect test is applied as the criterion of the nitrogen availability of a compound, it is apparent from Table VI that both allantoin and hydantoin served as a nitrogen source in a manner quite similar to uric acid. The nitrogen of hydantoin, however, appears less readily available than that of the other two compounds.

A more detailed study of the data for the previous experiments revealed that there was a difference in the

rapidity of acid production by the A. aerogenes and A. cloacae strains. In the allantoin medium (No. I) 92% of the 71 A. aerogenes strains had changed the reaction by the first day as compared to 49% of the 39 A. cloacae strains; however, all of the A. aerogenes strains acidified the medium by the third day and all of the A. cloacae strains by the fourth day. The contrast between these two species was even greater in the hydantoin medium (No. I). In this medium all of the A. aerogenes strains acidified the medium by the third day as compared to 60% of the A. cloacae strains; on the fifth day 15% of the A. cloacae strains still had failed to attack the glucose.

In the allantoin medium 21% of the Aerobacter strains reversed the acid reaction to the original alkalinity, or even more, by the fifth day; several strains reversed the reaction by the second day. In the hydantoin medium none of the Aerobacter strains reversed the reaction.

When vigor of growth was employed as the criterion of utilization of nitrogen, the similarity of the allantoin to the uric acid medium was again noted; hydantoin again appeared as a lesser available nitrogen source. Neither the Escherichia nor "intermediate" strains grew well; the VP (?) strains grew luxuriantly. A comparative study of the vigor of growth on the third day of the A. cloacae and A. aerogenes species appears in Table VII.

Table VII

Amount of Growth on Third Day

Species	Number of Strains	Allantoin as nitrogen source	Hydantoin as nitrogen source
		Per Cent Strains Growing Vigorously	
A. cloacae	39	31	0
A. aerogenes	72	100	96

The same 27 strains of A. cloacae which failed to grow vigorously in the uric acid medium (No. I) also failed to do so in allantoin medium (No. I). In hydantoin medium (No. I) 11 of the A. cloacae and two of the A. aerogenes showed poor growth.

Exploratory runs were made with allantoin and hydantoin to secure some indication as to their availability as carbon (Medium No. II), or both carbon and nitrogen (Medium No. III) sources for the colon group. Ten Escherichia, eight "intermediate," five A. cloacae and five A. aerogenes strains were employed as test organisms. The tabulated data of the five days' incubation showed that the Escherichia, the "intermediate," and the A. cloacae had neither grown well in, nor acidified either Medium II or III, or both compounds.

The five A. aerogenes strains grew vigorously when allantoin (Medium No. II) was the sole carbon source; two of these alkalinized the medium, two did not affect it, and one

acidified it. Similar results were secured when this compound was the source of both carbon and nitrogen (Medium No. III), except that the one strain which acidified the previous medium caused no change in reaction here.

Hydantoin (Medium No. II) as a carbon source supported the growth of the five A. aerogenes strains, three of which acidified the medium. When this compound was the source of both carbon and nitrogen, (Medium No. III) the five A. aerogenes strains grew slowly and created a heavy turbidity only on the fifth day; one strain acidified the medium.

In an exploratory run allantoin and hydantoin, Medium No. I, were subjected to moderate autoclaving. After inoculation and five days' incubation it was found that with both compounds the results for the ten Escherichia and ten Aerobacter test strains were the same as with the filtered medium. Of the "intermediates," however, five of six strains inoculated into allantoin, and four out of five strains inoculated into hydantoin had acidified the medium by the third day. It appeared that moderate autoclaving affected both allantoin and hydantoin in such a manner as to allow the "intermediate" strains to utilize the nitrogen, whereas the Escherichia were still unable to do so.

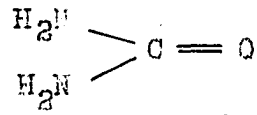
An exploratory run was made to secure an indication of the effect of heavy inoculation of Medium No. I when allantoin was used as the test compound. Five Escherichia strains were

inoculated into ten tubes; five tubes were heavily seeded from an agar slant, and the five others were inoculated in the usual manner with a one mm. loop of broth culture. All of the heavily inoculated tubes showed vigorous growth and had acidified the medium by the second day; the lightly inoculated tubes were negative in these two respects at the end of five days. It was evident that heavy inoculation permitted Escherichia strains to grow in allantoin(Medium No. I).

The availability of the nitrogen of allantoin to the colon group was similar to that of uric acid in that only Aerobacter and VP (?) strains were able to acidify the medium. The nitrogen of hydantoin was less available to the A. cloacae strains, but otherwise it behaved as allantoin.

Exploratory runs indicated that: (1) allantoin and hydantoin served as carbon, or both carbon and nitrogen sources for A. aerogenes only of the colon group studied, and thus were similar to nucleic and uric acids; (2) heavy inoculation could not be used in an investigation of these compounds as available nitrogen sources; and (3) moderate autoclaving affected both compounds in such a manner that "intermediate" strains thereafter utilized the nitrogen present.

d. Experiments with urea



urea

The urea went into solution readily.

When urea medium (No. I) was inoculated with 106 Escherichia, 137 "intermediate," 111 Aerobacter, and the six VP (?) strains, the tabulated data showed this compound to be an available source of nitrogen for Aerobacter, VP (?) and a high percentage of the "intermediate" strains, but for only two of the Escherichia strains. Thus it segregated the Aerobacter and "intermediate" strains together, as against the Escherichia strains. In this respect urea differed, as a nitrogen source, from all the compounds studied. The results of this experiment appear in Table VIII.

Table VIII

Acid Production from Glucose with Urea Supplied as Nitrogen Source

Group	Number of Strains	Per Cent of Strains Acidifying Medium				
		1 day	2 day	3 day	4 day	5 day
Escherichia	106	0	1	2	2	2
"Intermediate"	137	84	91	92	92	92
Aerobacter	111	91	99	99	100	100
VP (?)	6	100	100	100	100	100

Reversion was rapid. On the third day approximately 98% of the Aerobacter strains previously acidifying the medium had reversed the reaction to the original, or in the majority

of cases to a greater alkalinity than the uninoculated medium; by the fourth day 89% of the "intermediates" had behaved in a similar manner.

There was no significant difference in the action of the A. cloacae and A. aerogenes strains.

All strains which effected a change in the reaction, grew vigorously.

An exploratory run was made on urea medium (No. II) as a carbon source. The test organisms included ten Escherichia, eight Aerobacter, five A. cloacae and five A. aerogenes strains. Of these only the five A. aerogenes strains grew vigorously, and they increased the alkalinity of the medium. Similar results were secured with the same strains when urea medium (No. III) was tested as a source of both carbon and nitrogen.

Urea medium (No. I) was subjected to moderate autoclaving and then inoculated with ten Escherichia, seven "intermediate," and ten Aerobacter strains. All strains grew vigorously and acidified the medium by the second day. On the fifth day, however, all of the Aerobacter, four of the seven "intermediate," but none of the Escherichia had reversed the reaction to a greater alkalinity than the uninoculated medium. It was apparent that autoclaving had affected the urea in such a manner that Medium No. I would then support the growth of Escherichia strains. The fact that the "intermediate" strains were urea -N (+) and the Escherichia ones urea -N (-) offers

an explanation as to why autoclaving the allantoin and hydantoin, Medium No. I, caused these media subsequently to support the growth of "intermediate" but not Escherichia strains, if it is assumed that urea was split off from the parent compound and remained as such.

Urea was an available nitrogen source for all of the Aerobacter, 92% of the "intermediate," but only 2 of 106 Escherichia strains, as judged either by vigor of growth or by acidification of the medium. Reversion of the acid reaction was quite rapid. This compound, therefore, differed, in the above respects, from any other in the present study of nitrogen utilization by the colon group.

Exploratory runs indicated that urea as a carbon, or both carbon and nitrogen source was similar to nucleic acid, uric acid, allantoin, hydantoin, and uracil in that it supported the growth of A. aerogenes, only. A test run indicated that urea medium (No. I) was affected by moderate autoclaving to the extent that subsequent inoculation permitted vigorous growth of Escherichia strains.

D. Pyruvic Acid Fixation in a Glycerol-Peptone-Bisulphite Medium as a Differential Test

Any simple test that would differentiate the "intermediate" from the Escherichia and Aerobacter strains would be valuable.

Reynolds (1935) found that 44 of 57 "intermediate," 3 of 20 Aerobacter, but none of 15 Escherichia strains gave a qualitative test for pyruvic acid after 48 hours' incubation at 37° C. in a glycerol-peptone-bisulphite medium. Three additional strains of the 20 Aerobacter gave questionably positive tests. The bisulphite was assumed to fix chemically the intermediate product, pyruvic acid. He suggested that further investigation might lead to the establishment of conditions that would secure a sufficiently high correlation to be of differential value.

The purpose of the present investigation was to apply Reynolds' technique, under varying conditions of temperature and length of incubation, to a sufficiently large number of strains to ascertain the differential possibilities of the method.

The medium was prepared according to Reynolds and had the following composition:

Glycerol	2.0%
Peptone	0.5%
Sodium bisulphite	0.2%

10 ml. of 1-molar phosphate buffer (pH 7) per 100. ml. of medium.

This medium, after autoclaving at 15 pounds for 15 minutes, had a reaction of pH 6.8 - 7.0.

The presence of pyruvic acid was detected by the nitroprusside test of Simon and Piaux (1924). Five ml.

of medium were saturated with ammonium sulphate, and to this was added 0.5 ml. of freshly prepared 4.0% sodium nitroprusside and 0.5 ml. of concentrated ammonium hydroxide. The tubes were thoroughly shaken and allowed to stand for one-half hour before reading. The presence of pyruvic acid was indicated by a blue coloration of the entire liquid. The intensity of color was roughly proportional to the amount of the acid present. Control tests made with known dilutions of pyruvic acid showed that the blue color shaded into a dark green at the weaker concentrations. Simon and Piaux reported that only acetophenone interfered with the reaction.

In the present work inoculations were made into 18 mm. diameter test tubes containing aproximately five ml. of medium. Inoculations were made either from a 24 hour broth culture or from an agar slant.

Reynolds' original method, employing two days' incubation at 37° C., was investigated with a larger number of strains. The tabulated results of this experiment appear in Table IX. A. cloacae strains do not attack glycerol and were not considered. Only Escherichia strains which were known to attack glycerol with production of acid and gas were included.

Table IX

Pyruvic Acid Fixation - 2 Days - 37° C

Group	Escherichia	"intermediate"	A.aerogenes
No. of Strains	30	138	68
Per Cent Positive	0	69	66

The data of Table IX indicate that under such conditions the fixation of pyruvic acid distinguished some "intermediate" and A. aerogenes strains from glycerol (-) Escherichia strains, but that it was not a dependable criterion for differentiation between "intermediate" and A. aerogenes strains.

It was hoped that some difference might be brought out between the "intermediate" and A. aerogenes strains. For this reason the effect of length of incubation and temperature of incubation were investigated. Inoculations were made into five tubes of medium; each day one was removed for testing. The results of this experiment appear in Table X.

Table X.

Effect of Temperature and Period of Incubation on Fixation of Pyruvic Acid

Temperature of Incubation	Number of Strains	Per Cent of Strains Positive				
		1 day	2 day	3 day	4 day	5 day
		"intermediate"				
30° C.	47	55	75	77	77	77
37° C.	47	34	62	60	57	53
		Aerobacter aerogenes				
30° C.	71	13	4	9	-	16
37° C.	69	36	57	64	51	42

As judged by the intensity of color developed in the test, the amount of pyruvic acid produced by the "intermediate" strains, and fixed by the bisulphite, in the majority of cases was greater than that produced by the A. aerogenes.

It is evident that both temperature and period of incubation have a decided effect on this test. Table X shows that a temperature of 30° C. favored securing the highest percentage of positive results with the "intermediate" and the lowest percentage of positive results with the A. aerogenes strains, while the reverse was true at 37° C.; after 3 days' incubation at 30° C., 77% of the "intermediates" gave a positive test as compared to 9% of the Aerobacter strains; a 37° C.

temperature led to a gradual decrease in the per cent of positive tests after the second or third day; the A. aerogenes strains gave irregular results at the lower temperature.

The daily tests of the individual strains varied more than the summarized data of Table X indicates. To illustrate this excerpts from different protocols are given below.

Temperature of incubation:	30° C.					37° C.				
Period of incubation	Days 1	2	3	4	5	Days 1	2	3	4	5
Strain	"intermediate"									
104-S	+	+	+	+	+	-	+	-	-	+
110-B	-	-	-	-	-	-	-	-	-	-
113-A	+	+	+	+	+	-	-	-	-	+
141 L-1	+	+	+	+	+	-	-	+	+	-
144-S	+	+	+	+	+	+	+	+	+	+
147	+	+	+	+	+	+	+	+	+	+
148	-	+	+	+	+	-	+	-	+	+
152A	+	+	+	+	+	-	+	+	+	+
158	+	+	+	+	+	-	-	+	+	-
165-1AA	+	+	+	+	+	-	+	+	-	+
	A. aerogenes									
469	-	-	-	-	-	-	+	-	+	-
470	-	-	-	-	-	-	-	+	+	+
471	-	-	-	-	+	+	-	+	-	-
472	-	-	-	-	-	+	+	+	+	-
473	+	-	+	+	-	+	+	+	+	-
474	+	-	-	+	-	-	+	+	+	+
475	+	-	-	-	-	+	+	+	+	+
476	-	-	-	-	-	-	-	+	+	+
477	-	-	-	-	-	-	+	-	-	+
478	-	-	-	-	-	-	+	+	+	+

Such irregular results make a standard incubation period,

which would be necessary for a simple differential test, a difficult, if not impossible, factor to establish.

It is regretted that time did not permit of an investigation of still lower temperatures, such as 25° or 20° C.

To summarize: an investigation of a large number of cultures showed that pyruvic acid fixation in a glycerol-peptone-bisulphite medium at 37° C. yielded positive results for approximately 68% of both "intermediate" and A. aerogenes, but the results were negative for all the glycerol (+) Escherchia strains tested. It, therefore, served as a confirmatory test in separating some of the "intermediate" strains from glycerol (+) Escherichia, but not from A. aerogenes strains. Both temperature and period of incubation had a decided effect on the probability of securing positive tests with both "intermediate" or A. aerogenes strains. At 30° C. three days' incubation resulted in 77% of the "intermediate" strains giving a positive test, as compared to 9% for the A. aerogenes strain. Due to the variability of results for the same organism from day to day the establishment of standard conditions for a routine test would be a difficult thing to accomplish.

III. DISCUSSION

Bodansky (1934) reviews the work of different investigators on the digestion of nucleic acid by warm blooded animals. He considers the process to occur as follows:

1. The nucleic acid is disintegrated to mononucleotides by a specific enzyme, polynucleotidase. Mononucleotides are composed of phosphoric acid, sugar, and a purine or pyrimidine base.

2. The nucleotides are hydrolysed by a non-specific enzyme, nucleotidase, to phosphoric acid and nucleosides. Nucleosides are composed of sugar and a purine or pyrimidine base.

3. The purine nucleosides are hydrolysed by nucleosidase into purine bases and a reducing sugar.

4. The pyrimidine nucleosides are completely metabolised to urea in some cases, although less is known of this process than of purine metabolism. The end product of uracil and thymine is probably urea.

The further transformation of the purine bases, and the enzymes concerned, is outlined below.

adenine + H_2O —(adenase)→ hypoxanthine + NH_3

hypoxanthine + O —(xanthine oxidase)→ xanthine

guanine + H_2O —(guanase)→ xanthine + NH_3

xanthine + O —(xanthine oxidase)→ uric acid

uric acid + O + H_2O —(uricase)→ allantoin + CO_2

Uric acid is the end product of purine metabolism in man, anthropoid apes, and the Dalmatian coach hound. Allantoin is reported the chief end product in other animals.

The in vitro chemical transformation of allantoin occurs as follows:

allantoin + H_2O ⟶ allanturic acid + urea

allanturic acid + H_2 ⟶ hydantoin

hydantoin + H_2O ⟶ hydanuric acid

hydanturic acid + H_2O ⟶ glycine + CO_2 + NH_3

That some of the coli-aerogenes strains possess certain of these enzymes is evident from their ability to attack the individual compounds. In Table XI is presented a summary of the studies on nitrogen availability together with other characteristics of the colon group. The individual reactions

are included for a transfer of the original Brenk strain which Werkman and Gillen (1932) designated Cit. Freundii and type species of the genus Citrobacter.

Table XI. Summary of Differential Characteristics of Colon Group

Group	Escherichia	Intermediate	Aerobacter	VP (?)	Cit. Freundii
Number of Strains	106	138	111	6	1
Reaction	Per Cent of Strains Giving Positive Reaction				
Yeast nucleic acid	0	0	96	100	-
Uric acid	0	0	100	100	-
Allantoin	0	0	100	100	-
Hydantoin	0	0	87	100	-
Uracil	80	2	100	100	-
Urea	2	92	100	100	+
VP	0	0	100		-
Citric acid	0	100	100	100	+
MR	100	95	0	100	+
Cellobiose	2	95 *	99	100	acid no gas
H_2S	1	83	0	0	+
Indol	98	6	23	17	-

* Of the 131 "intermediate" strains which attacked cellobiose, 97 produced acid but no gas.

As the nitrogen of xanthine and adenine sulphate was utilized by all colon strains tested, these compounds are not listed. A study of Table XI shows that on the basis of utilization of nitrogen compounds the colon group may be separated into three divisions; one which utilizes all of these compounds, a second which utilizes only uracil, and a third which utilizes only urea. From the results of Table XI a key may be formed for these divisions.

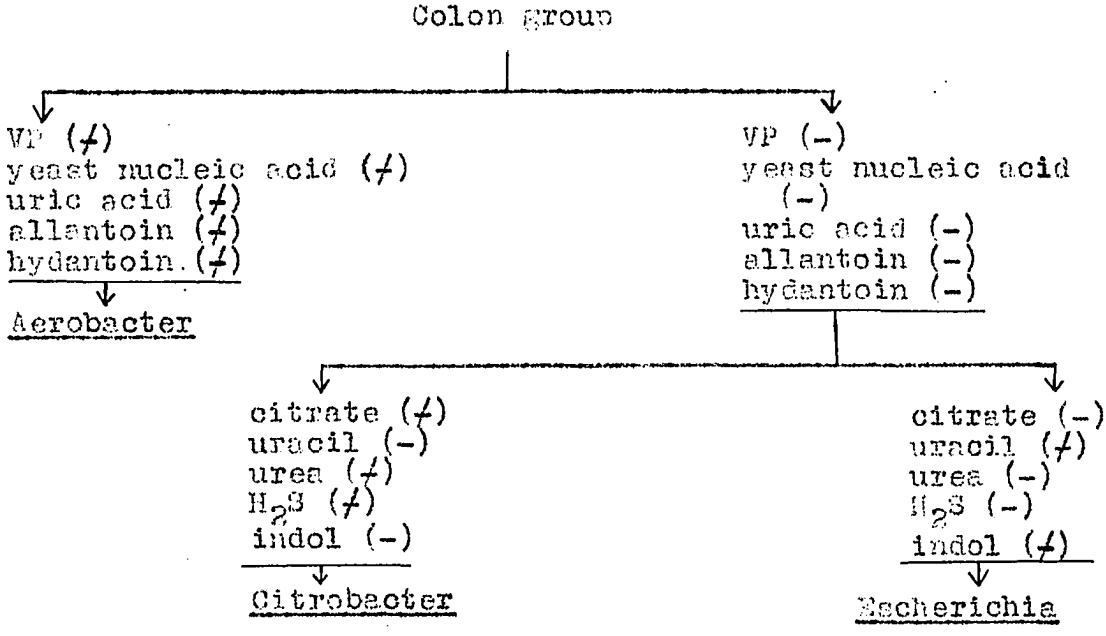


Table XI shows the percentage of strains which gave the reactions listed for the key. It is felt that such a study shows the "intermediate" strains to be a sufficiently unified group within itself and distinct from the genera Aerobacter and Escherichia to warrant recognition as a genus. It is

evident that the majority of the VP (-), citrate (+) strains behave as Citrobacter Freundii, the type species of the genus. The H_2S (Vaughn and Levine medium) and uracil tests are especially helpful for simple and rapid identification of Citrobacter strains.

Bodansky states that "certain organisms of the alimentary tract are said to be capable of synthesizing purines and even uric acid." On the other hand Lucke (1930, 1931) presents evidence that a great part of the uric acid of the gastric juice and bile is destroyed in the alimentary canal. He finds that this destruction commences at the region in the small intestine where the bacterial flora begins to show great numbers. It is known that Aerobacter organisms predominate in the upper small intestine. Since the present investigations show that Aerobacter strains quite readily attack nucleic acid and certain of its degradation products, it is logical to assume that any nucleic acid in the intestines would be disintegrated by bacteria as well as by the digestive enzymes of the host.

It is regretted that time did not permit of a more detailed chemical study of the fermentations. Since the methods of qualitative and quantitative determination of many of these compounds already have been worked out in physiological chemistry, such a study of bacterial fermenta-

tion would be greatly facilitated. That the Aerobacter and VP (?) strains are able to utilize all of the nitrogen compounds studied lends support to a hypothesis that the disintegration of nucleic acid by these strains may be analagous to that occurring in animal digestion. If that is true, then a study of the bacterial fermentation of these compounds offers a readily available method of investigating these processes.

It would be interesting to know the point at which these nitrogen compounds are attacked. The Aerobacter strains utilize all compounds tested as nitrogen sources and therefore offer no clue. That the Escherichia and "intermediate" strains could not attack the imidazole ring is suggested by their failure to attack allantoin and hydantoin. That the imidazole ring attached to urea is inhibitory to the "intermediate" strains, whereas urea itself is an available nitrogen source, is suggested by the failure of this group to attack allantoin. Is it then the imidazole ring of the purine compound which is the factor prohibiting the utilization of the nitrogen of uric acid? That Escherichia strains attack the pyrimidine uracil lends support to this hypothesis.

The question may then be asked as to why xanthine (Chenn's, et al. report to the contrary) and adenine sulphate are utilized as nitrogen sources. The only apparent difference in the imidazole ring of xanthine as compared to that of

uric, allantoin and hydantoin is that in the former compound nitrogen is attached by double bonds to a carbon, whereas in the latter three compounds it is by a single bond. The evidence for such an explanation is slight, and the study of other imidazole ring compounds as nitrogen sources would be necessary before such a statement could be accepted as anything other than as a hypothesis.

As to the utilization of the nitrogen of adenine sulphate, it is to be born in mind that various radicals may greatly effect a compound's utilization by bacteria, e.g. glucose and alpha-methylglucoside are not attacked with the same ease by the colon group, methyl-urea and thio-urea are not equally available nitrogen sources to the same bacteria (de Jong). It may be that the sulphate radical makes the adenine more easily attacked.

The bacteriological studies herein reported show that the nitrogen availabilities of this related group of compounds may serve to differentiate generic groups. Since the VP (?) strains behaved as the Aerobacter strains with respect to every compound listed, it would seem logical to allocate them to this genus. It appears that studies on the utilization of nitrogen of nucleic acid and its degradation products may serve as a substantial aid in the allocation of strains of the colon group which give questionable VP tests.

The genus Citrobacter is accorded generic ranking with Escherichia and Aerobacter.

IV. SUMMARY

1. The colon group was studied to determine if nitrogen utilization was as distinctive for generic differentiation as is carbohydrate utilization and dissimilation. Nucleic acid and its degradation products (which occur in animal digestion and also in in-vitro chemical transformation) were employed as nitrogen sources.

2. A synthetic medium was employed in which glucose served as a carbohydrate source. All such media were sterilized by filtration. Two criteria of nitrogen utilization were employed: acid production from glucose and vigor of growth. The cultures studied included 106 Escherichia, 138 Citrobacter, 39 A. cloacae, 71 A. aerogenes, and 6 strains which gave a questionable VP test.

3. With very few exceptions the results tabulated below were obtained for the utilization of the compounds as nitrogen sources after four days' incubation at 30° C.

Group	Yeast Nucleic Acid	Uric Acid	Allantoin	Hydantoin	Uracil	Urea	Adenine SO_4	Xanthine
Escherichia	-	-	-	-	≠	-	≠	≠
Citrobacter	-	-	-	-	-	≠	≠	≠
Aerobacter	≠	≠	≠	≠	≠	≠	≠	≠
VP (?)	≠	≠	≠	≠	≠	≠	≠	≠

4. Uracil appears to have differential value for separating the Citrobacter genus from the genera Aerobacter and Escherichia on the basis of nitrogen utilization. Further work on nitrogen availability of pyrimidine compounds may prove fruitful.

5. The Escherichia strains differed from all others in that they were unable to utilize the nitrogen of urea.

6. Since the six VP (?) strains utilized the nitrogen of all compounds, as did the Aerobacter strains, they are allocated to the genus Aerobacter. Information on the nitrogen utilization may be an aid in allocation of colon strains giving questionable VP reactions.

7. Exploratory runs with a limited number of strains indicated that A. aerogenes could utilize most of the compounds studied as sole sources of carbon, or of carbon and nitrogen, but that Escherichia, Citrobacter, and A. cloacae strains could not.

8. Exploratory runs with a limited number of strains indicated that of the compounds studied only allantoin, hydantoin, and urea were affected by moderate autoclaving.

9. Pyruvic acid fixation in a glycerol-peptone-bisulphite medium was not found to be a dependable criterion for differentiation between Citrobacter and A. aerogenes strains.

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Appendix

TABLE A

Individual Characteristics of Citro

CF Designates *Citrobacter Fre*

"INTERMEDIATE" STRAI

Reactions	1A	1Bb	3	4RG	7B②	8B	9RG	14B	14RGD	14RGL	16B	17D	19B	22B	23B	27A	27B	29	29B	30RG	33D	33RG	33-1	33-2	33-3	35C	36RG	37	38	40A	40B	45-2	55A	55G	58S	79B	81A	81B	96	104S
V.P.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M.R.	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
H_2S	+	+	+	+	+	+	+	−	−	−	+	+	+	−	−	+	+	+	+	−	−	−	+	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+
Indol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Cellobiose	+	+	+	+	⊕	+	+	+	+	+	+	+	+	+	+	⊕	+	+	+	⊕	+	+	⊕	⊕	⊕	−	+	⊕	⊕	+	+	+	+	+	−	+	+	+	+	+
Nucleic Acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Xanthine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Uric Acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Adenine Sulfate		+			+	+								+											+	+									+					
Uracil	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Allantoin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Hydantoin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Urea	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+

"INTERMEDIATE" STRAINS - CIT

Reactions	311S	360S	410	503-SA	511	515S	516S	529	542S	557S	590	596S	601	691	729	739	749	Bard 1	Bard 1a	Bard 2	Bard 3	Bard 3a	Bard 4	Bard 4a	Bard 5	Bard 5A	Gr III A	Gr III B	V5	V16A	V22A	V26	V27	X26	CF	H1	H2	H3	H4	H5
V.P.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M.R.	+	+	+	±	+	+	−	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
H_2S	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+
Indol	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
Cellobiose	+	−	+	+	⊕	+	⊕	+	⊕	+	+	+	⊕	+	+	+	+	+	+	+	⊕	⊕	−	+	+	+	+	+	⊕	⊕	⊕	⊕	⊕	+	+	⊕	+	⊕	⊕	+
Nucleic Acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Xanthine																																								
Uric Acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Adenine Sulfate																																					+			
Uracil	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Allantoin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Hydantoin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Urea	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Cellobiose: ⊕ denotes acid and gas production.
+ denotes acid but not gas production.
− denotes neither acid nor gas production.

TABLE A

cteristics of Citrobacter Strains

signates *Citrobacter Freundii*

ERMEDIATE" STRAINS – CITROBACTER

38 40A	40B 45-2 55A 55G 58S	79B 81A 81B 96 104S	110B 113A 113B 123B 128C	139S 141L-1 141L-2 144S 147	148 149S 152A 152B 158	163-CA 163-CB 165-1AA 165-1AB 165-1B	165-2A 165-2B 176 194-S 201	202 217 250 277 278	282-1 282 292 295L 308-S
– –	– – – – –	– – – – –	– – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – –
+ –	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ ± + + +	+ + + + +	+ + + + +	– – + + +
– +	+ + + + +	+ + + + +	– + + + +	– + + + +	+ + – – +	+ + + + +	+ + + + +	+ + + + +	+ + + + +
– –	– – – – +	– – – – –	+ – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – –
⊕ +	+ + + + –	+ + + + +	– + + + +	+ + + – +	+ + + + +	+ + + + ⊕	+ + + + ⊕	+ ⊕ + + +	+ + ⊕ + +
– –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – –
+ +	+ + + + +	+ + + + +	+ + + + +	+ + + +					
– –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – –
	+								
– –	– – – – –	– – – – –	+ – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – –
– –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – –
– –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – –
+ +	+ + + + + –	+ + + + +	– + + + +	+ + + + + +	+ – + + +	+ + + + + +	+ + + + +	– + + + +	+ + + + +

ATE" STRAINS – CITROBACTER

V5 V16A	V22A V26 V27 X26 CF	H1 H2 H3 H4 H5	C12 C23 C26 C33 C46	C53 C73 C79 C83 C84	C105 C107 C112 C114 C116	C119 GHMESD Int.A Int.B 37RG	88 107D 287
– –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – –
+ +	+ + + + +	+ + + – –	+ + + + +	+ + + + +	+ + + +	± + + + +	+ + +
– –	– – + + +	+ + + + +	+ + + + –	– + + + +	+ + + + +	+ + + + +	+ – +
+ +	+ + – – –	– – – – –	– – – + –	– – – – –	– – – – –	– – – – –	– + –
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V.P.(?) STRAINS

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roduction.
ns production.
gas production.

All other tests: + denotes positive reaction.
– denotes negative reaction.